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1) 0942716 PMID: 11084331
Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype.
Uren A G; Wong L; Pakusch M; Fowler K J; Burrows F J; Vaux D L; Choo K H
The Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, 3050,., Victoria, Australia.
Current biology - CB (ENGLAND) Nov 2 2000, 10 (21) p1319-28, ISSN 0960-9822 Journal Code: 9107782

2) 1419888 PMID: 11516652
INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis.
Wheatley S P; Carvalho A; Vagnarelli P; Earnshaw W C
Chromosome Structure Group, Institute of Cell and Molecular Biology, Swann Building, University of Edinburgh, King's Buildings, Mayfield Road, EH9 3JR, Edinburgh, United Kingdom.
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3) ####14149991 PMID: 9850056
IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs.
Tamm I; Wang Y; Sausville E; Scudiero D A; Vigna N; Oltersdorf T; Reed J C
The Burnham Institute, La Jolla, California 92037, USA.
Cancer research (UNITED STATES) Dec 1 1998, 58 (23) p5315-20, ISSN 0008-5472 Journal Code: 2984705R
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4) ###10863651 PMID: 10997903
Crystal structure and mutational analysis of the Saccharomyces cerevisiae cell cycle regulatory protein Cks1: implications for domain swapping, anion binding and protein interactions.
Bourne Y; Watson M H; Arvai A S; Bernstein S L; Reed S I; Tainer J A
Centre National de la Recherche Scientifique, Marseille, France. yves@afmb.cnrs-mrs.fr
Structure with Folding & design (ENGLAND) Aug 15 2000, 8 (8) p841-50 , ISSN 0969-2126 Journal Code: 100889329
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5) tle: P35 IS A NEURAL-SPECIFIC REGULATORY SUBUNIT OF CYCLIN-DEPENDENT KINASE-5 (Abstract Available)
Author(s): TSAI LH; DELALLE I; CAVINESS VS; CHAE T; HARLOW E
Corporate Source: HARVARD UNIV,SCH MED,DEPT PATHOL,200 LONGWOOD AVE/BOSTON//MA/02115; HARVARD UNIV,MASSACHUSETTS GEN HOSP,SCH MED,DEPT NEUROL/BOSTON//MA/02114; MASSACHUSETTS GEN HOSP,CTR CANC/BOSTON//MA/02129
Journal: NATURE, 1994, V371, N6496 (SEP 29), P419-423

6) $$$ Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1.
Bourne Y; Watson M H; Hickey M J; Holmes W; Rocque W; Reed S I; Tainer J A

INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis

Sally P. Wheatley*, Ana Carvalho*†, Paola Vagnarelli* and William C. Earnshaw*

Three lines of investigation have suggested that interactions between Survivin and the chromosomal passenger proteins INCENP and Aurora-B kinase may be important for mitotic progression. First, interference with the function of Survivin/BIR1, INCENP, or Aurora-B kinase leads to similar defects in mitosis and cytokinesis [1–7] (see [8] for review). Second, INCENP and Aurora-B exist in a complex in *Xenopus* eggs [9] and in mammalian cultured cells [7]. Third, interference with Survivin or INCENP function causes Aurora-B kinase to be mislocalized in mitosis in both *C. elegans* and vertebrates [5, 7, 9]. Here, we provide evidence that Survivin, Aurora-B, and INCENP interact physically and functionally. Direct visualization of Survivin-GFP in mitotic cells reveals that it localizes identically to INCENP and Aurora-B. Survivin binds directly to both Aurora-B and INCENP in yeast two-hybrid and in vitro pull-down assays. The in vitro interaction between Survivin and Aurora-B is extraordinarily stable in that it resists 3 M NaCl. Finally, Survivin and INCENP interact functionally in vivo; in cells in which INCENP localization is disrupted, Survivin adheres to the chromosomes and no longer concentrates at the centromeres or transfers to the anaphase spindle midzone. Our data provide the first biochemical evidence that Survivin can interact directly with members of the chromosomal passenger complex.

Addresses: *Chromosome Structure Group, Institute of Cell and Molecular Biology, Swann Building, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR, United Kingdom. †University of Porto, Instituto de Patologia e Imunologia Molecular da Universidade do Porto, R. Dr. Roberto Fias, S/N, 4200 Porto, Portugal.

Correspondence: William C. Earnshaw
E-mail: bill.earnshaw@ed.ac.uk

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Results and discussion

Human Survivin is a bona fide chromosomal passenger protein

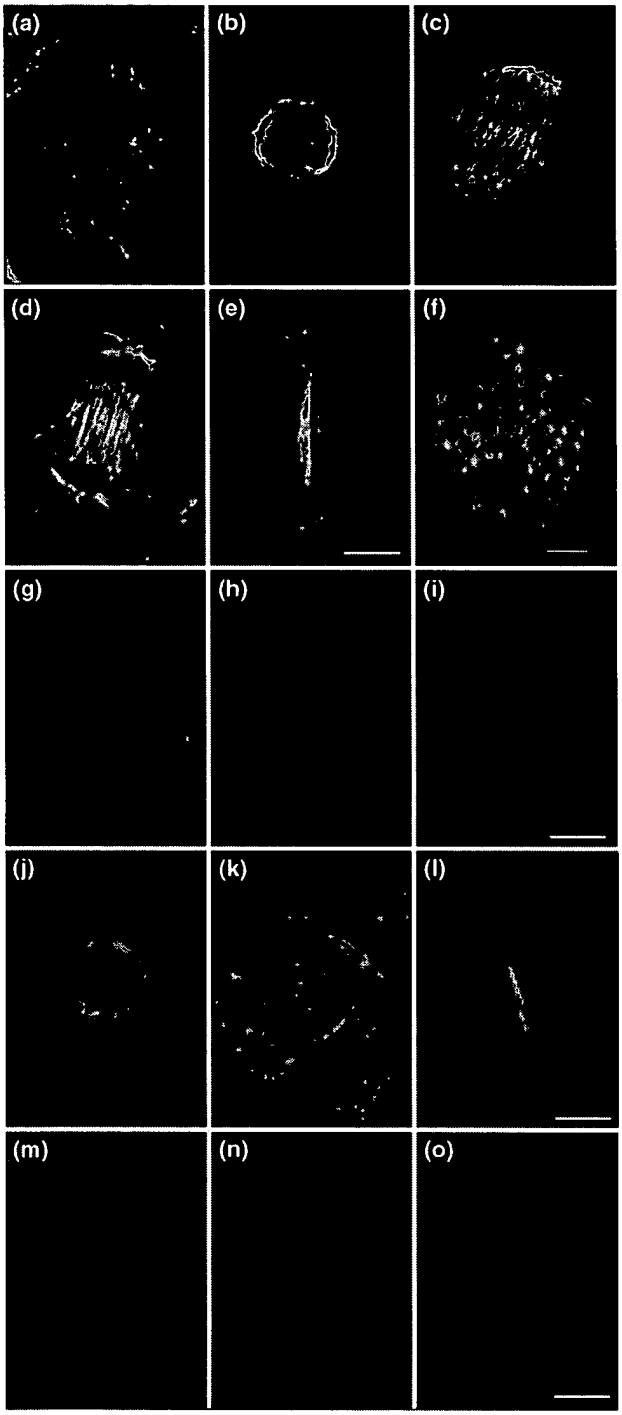
Recent attempts to determine the role of Survivin in the regulation of mitosis and apoptosis have been hindered by a controversy over the protein's localization in mitotic cells. The original studies found Survivin to be associated with spindle microtubules and suggested that this association was required for its anti-apoptotic function [10, 11]. In contrast, recent studies for which different antibodies were used have suggested that Survivin is a chromosomal passenger protein [12] that associates with chromosomes early in mitosis and transfers to the spindle at ana/telophase [13, 14].

Here we used two antibodies to localize Survivin in mitotic cells. Staining of HeLa cells with a commercial polyclonal antibody (www.novus-biologicals.com) indicated that Survivin associates with chromosomes early in mitosis and is released from the centromeres to the central spindle and equatorial cortex during anaphase (Figure 1a–e). In cytokinesis, Survivin accumulates in the midbody and is eventually discarded with the intercellular bridge. This pattern of localization was also seen in human osteosarcoma (U2OS) and chicken hepatoma (DU249, not shown) cells and was completely blocked when the antibody was preincubated with GST-Survivin (Figure 1g–i). Chromosomal spreads from mitotic HeLa cells (Figure 1f) and costaining with anti-Survivin and anti-Aurora-B antibodies (Figure 4a,b,e) revealed that Survivin and Aurora-B colocalize on centromeres at metaphase and at the midzone in anaphase. This behavior is characteristic of the chromosomal passenger proteins [3, 12, 15–17].

When we probed mitotic cells with the 8E2 monoclonal antibody used in the original immunolocalization studies of Survivin [10, 11], we obtained a strikingly different result. Survivin was observed on microtubules throughout mitosis, with no binding to chromosomes, centromeres, or the spindle midzone (Figure 1j–l). In controls, preabsorption of 8E2 with GST-Survivin completely abolished the staining pattern (Figure 1m–o).

To resolve this conflict, we visualized Survivin directly in mitotic HeLa cells transiently transfected with C-terminally GFP-tagged Survivin cDNA. Survivin-GFP exhibited a chromosomal passenger distribution identical to that obtained by immunofluorescence with the commercial antibody (Figure 2a–f). This distribution was also ob-

Figure 1



Conflicting Survivin localization in mitosis with two published antibodies. **(a–e)** HeLa cells were probed for Survivin with a rabbit polyclonal anti-Survivin antibody (www.novus-biochemicals.com; red), anti-tubulin (green), and DAPI (blue). (a) Survivin is not associated with the chromosomes in early prophase, (b) is centromeric during prometaphase and metaphase, (c,d) localizes to the midzone and equatorial cortex during anaphase and telophase, and (e) localizes to the midbody during cytokinesis. **(f)** Mitotic HeLa cells were spread and probed simultaneously for Survivin (red) and DNA (blue). Survivin localizes at centromeres. **(g–i)** Preincubation of the antibody with a 10-fold excess of GST-Survivin for 1 hr at 25°C completely blocked the staining. **(j–l)** A mouse monoclonal antibody, 8E2 [10], shows Survivin (red) to be localized on microtubules throughout mitosis: (j) metaphase, (k) anaphase, (l) cytokinesis. **(m–o)** Preincubation of 8E2 antibody with GST-Survivin also completely abolished staining. The scale bars represent 5 μm.

served in cells stably expressing the chimeric protein (data not shown). Centromeric and midbody staining was also seen with Survivin tagged at its NH2 terminus with HA-His6, or CFP; however, transfer to the anaphase central spindle was not observed (data not shown).

The reason for the different localization observed with the original anti-Survivin monoclonal antibody is not known, but it could be due to the recognition of a splice variant or a shared epitope present on another spindle-associated protein. Regardless, we can now be confident that human Survivin is a bona fide chromosomal passenger protein as claimed by [13, 14].

Survivin interacts directly with Aurora-B and INCENP

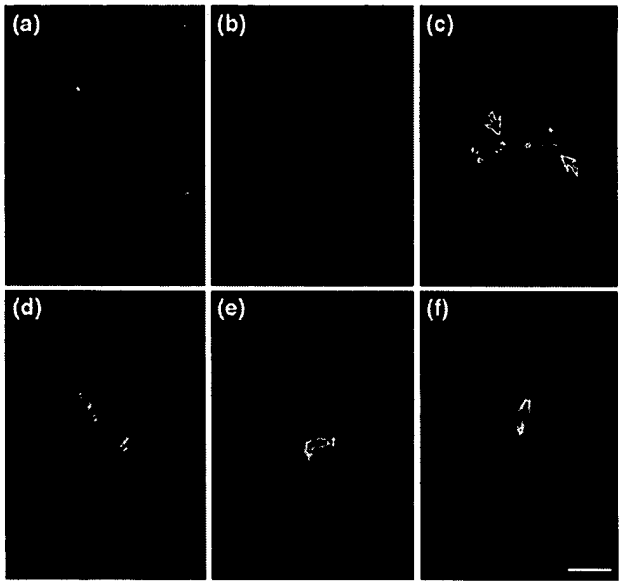
A key question raised by recent studies of chromosomal passengers (for review, see [8]) is whether Survivin, Aurora-B, and INCENP interact directly with one another. Using the yeast two-hybrid system, we detected an extremely robust interaction between Survivin and Aurora-B kinase and a significant interaction between Survivin and INCENP (Figure 3a). The interaction with Aurora-B was an order of magnitude stronger than that between INCENP and the positive control, heterochromatin protein-1 (HP1; [18]).

We confirmed a direct interaction between Survivin and Aurora-B on the one hand and between Survivin and INCENP on the other by using a GST-fractionation assay. In vitro-translated INCENP and Aurora-B bound specifically to glutathione sepharose beads coated with GST-Survivin (Figure 3b,c, lane 4) or GST-Survivin-GFP (Figure 3b,c, lane 8), but not GST alone (Figure 3b,c, lane 2) or, in the case of Aurora-B, GST-HP1 (Figure 3b, lane 6). These in vitro interactions are extremely robust; the interaction between Survivin and Aurora-B was stable even at 3 M NaCl (Figure 3d), and Survivin and INCENP maintain their association up to 0.6 M NaCl (Figure 3d). These data verify that Survivin can bind directly to both Aurora-B and INCENP and demonstrate that Survivin-GFP behaves similarly to untagged Survivin in vitro.

A dominant-negative INCENP mutant blocks Survivin localization to centromeres and the spindle midzone

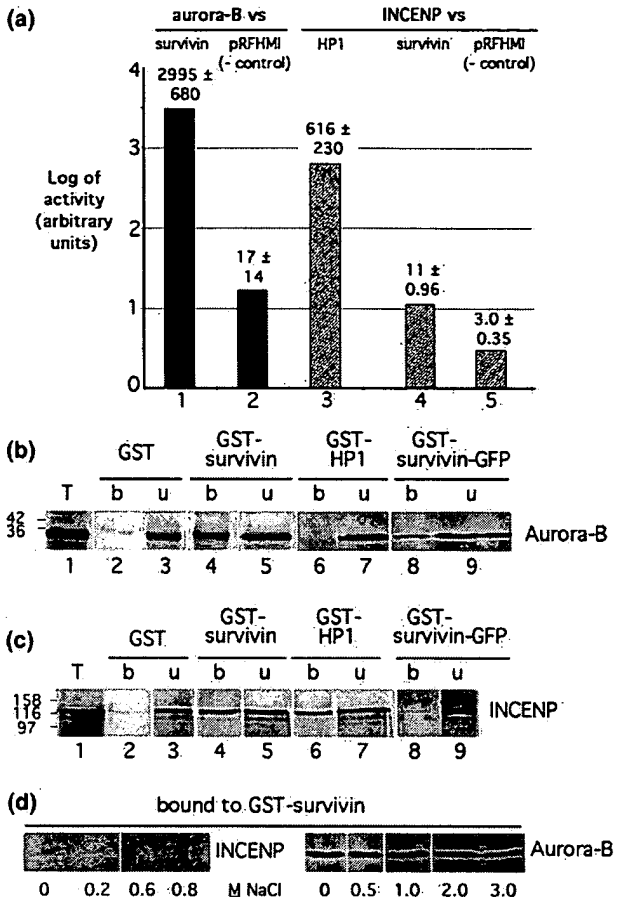
We obtained conclusive evidence for a functional interaction between Survivin and INCENP in vivo by using the dominant-negative INCENP mutant, $INCENP_{1-405}$ [2].

Figure 2



Human Survivin-GFP is a chromosomal passenger in HeLa cells. HeLa cells were transiently transfected by electroporation with C-terminally GFP-tagged human Survivin and were observed after 24 hr. Survivin-GFP was **(a)** cytoplasmic in interphase, **(b)** interchromosomal in prophase, and localized on **(c)** the centromeres during prometaphase/metaphase, **(d,e)** the spindle midzone and the equatorial cell cortex during anaphase/telophase, and **(f)** the midbody during cytokinesis. Arrowheads in (c) point to centromeres. The scale bars represent 5 μm.

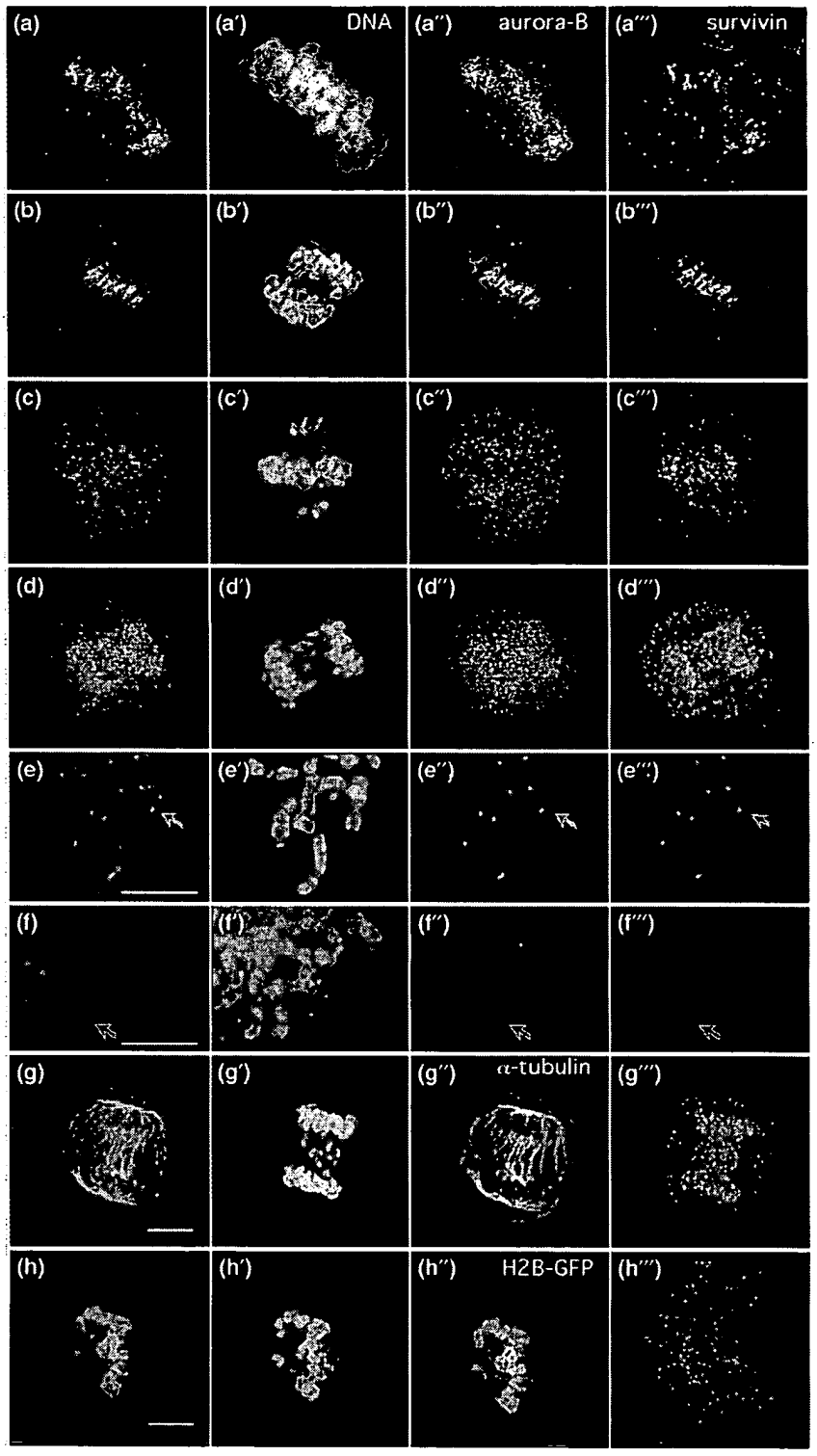
Figure 3



In transfected cells expressing $INCENP_{1-405}$, endogenous INCENP fails to localize correctly [2], and Aurora-B is distributed diffusely throughout the cytoplasm [9]. $INCENP_{1-405}$ also disrupted Survivin localization in mitotic cells, though in a manner distinct from its effect on Aurora-B kinase (Figure 4). In 90% of prometaphase HeLa cells transiently transfected with $INCENP_{1-405}$ (N = 161; Figure 4c), Survivin was distributed evenly along the chromosome arms and did not concentrate at the centromeres. The remaining 10% of transfected cells showed some concentration of Survivin at the centromeres, and this observation was possibly indicative of the presence of residual wild-type INCENP or Aurora-B protein. During anaphase/telophase, despite the development of a normal central spindle (Figure 4g), Survivin did not transfer to the central spindle or the equatorial cortex but remained on the chromosomes in 80% of cells transfected with $INCENP_{1-405}$ (N = 20); 20% of cells showed a more diffuse pattern of staining. In colcemid-blocked cells, $INCENP_{1-405}$ also prevented Survivin-GFP from targeting to centromeres (Figure 4e,f). Therefore, INCENP is required for targeting Survivin to its centromeric and central spindle locations during mitosis and may lie upstream of Survivin in a common pathway. Interestingly,

Survivin interacts directly with Aurora-B and INCENP. **(a)** Human Survivin cDNA in the two-hybrid bait vector pEG202 was tested for interaction with full-length human Aurora-B (lane 1) or chicken INCENP (lane 4) in pJG4.5 with a quantitative β-galactosidase assay as described previously [21]. Activity is plotted on a log scale. pRFHMI versus Aurora-B or INCENP is shown in lanes 2 and 5 (negative control). Lane 3 shows the interaction between INCENP and heterochromatin protein-1 (HP1; positive control). This assay was negative, with the vectors in the opposite orientation. **(b,c)** Human Survivin interacts directly with Aurora-B and INCENP in vitro. Survivin fused at its NH_2 terminus to GST was bound to glutathione sepharose beads and incubated with (b) in vitro-translated Aurora-B or (c) INCENP. The lanes are as follows: lane 1, Total input (T); lane 2, material bound to GST alone (In the negative control, GST was used in approximate 3-fold excess over the GST-Survivin); lane 3, supernatant for GST alone; lane 4, GST-Survivin pellet; lane 5, GST-Survivin supernatant; lane 6, GST-HP1 pellet; lane 7, GST-HP1 supernatant; lane 8, GST-Survivin-GFP pellet; lane 9, GST-Survivin-GFP supernatant. Bound (b) and unbound (u) fractions are labeled. Both Aurora-B and INCENP bound to beads coated with GST-Survivin and GST-Survivin-GFP (lanes 4 and 8). **(d)** GST-Survivin-coated beads were incubated with in vitro-translated INCENP (left) or Aurora-B (right), then washed for 1 hr in NaCl as indicated. The interaction between GST-Survivin and Aurora-B was extremely robust and withstood 3 M NaCl, while INCENP remained bound to GST-Survivin at 600 mM NaCl.

Figure 4



$INCENP_{1-405}$ prevents Survivin from targeting to the centromeres and the central spindle. HeLa cells were transiently transfected by electroporation with the dominant-negative INCENP mutant, $INCENP_{1-405}$ [2]. After 24 hr, cells were fixed, counterstained for DNA with DAPI (blue, "prime" panels), and probed for Aurora-B with the monoclonal antibody anti-AIM1 (green, "double prime" panels); cells were probed for Survivin by the use of Novus polyclonal antibody (red, "triple prime" panels), and images were processed as described above. Aurora-B mislocalization was used for the identification of transfected cells (see [9]). In control cells, Aurora-B and Survivin localized to **(a–a‴)** centromeres during prometaphase and metaphase and to **(b–b‴)** the central spindle and equatorial cortex during anaphase. **(c–c‴,d–d‴,g–g‴)** In cells transfected with $INCENP_{1-405}$, Aurora-B was dispersed throughout the cell in prometaphase and anaphase ("double prime" panels), while Survivin bound all along the chromosomes and did not transfer to the centromeres, central spindle, or the equatorial cortex ("triple prime" panels). **(e–e‴,f–f‴)** Transfection with $INCENP_{1-405}$ also blocks Survivin-GFP from targeting to centromeres (arrows). (e–e‴) An untransfected cell blocked with colcemid shows accumulation of (e‴) Survivin-GFP and (e″) Aurora-B at the centromeres. (f–f‴) Cell transfected with $INCENP_{1-405}$ and blocked with colcemid. (f‴) Survivin-GFP and (f″) Aurora-B are no longer at the centromeres. Note that the exposure in panels (g″) and (g‴) is elevated three times relative to that in panels (f′) and (f‴). Panels (g–g‴) show that a normal central spindle forms between the separating chromosomes in $INCENP_{1-405}$-transfected cells; (g″) microtubules, (g‴) Survivin. **(h–h‴)** Cotransfection with a plasmid expressing (h″) H2B-GFP (green) and $INCENP_{1-405}$ confirms that (h‴) Aurora-B/AIM1 (red) is diffuse in cells transfected with $INCENP_{1-405}$. The scale bar represents 5 μm.

it was recently reported that a Survivin mutant that disrupts the function of the BIR domain causes Survivin to become spread diffusely throughout the cell, but TD60 is localized correctly [14]. These findings suggest that either those two proteins act in different pathways or that TD-60 is also upstream of Survivin.

The pattern of Survivin disruption observed in cells transfected with $INCENP_{1-405}$ mimics the behavior of *Drosophila* INCENP in cultured cells when the Aurora-B kinase is abolished by dsRNA-mediated interference (RNAi). DmINCENP was observed to associate with chromosomes but to target poorly, if at all, to centromeres and not to transfer to the spindle [19]. This raises the possibility that Survivin localization to centromeres and the central spindle could be regulated by Aurora-B kinase. However, Survivin is apparently not a substrate of Aurora-B kinase (R.R. Adams, S.P.W., W.C.E., unpublished data) and may instead be regulated by Cdk1:cyclin B1 [20].

Despite profoundly disrupting mitosis, including the localization of Survivin, INCENP, and Aurora-B kinase, $INCENP_{1-405}$ does not cause an increase in apoptosis. This is important because we did not observe any association of Survivin with spindle microtubules in this mutant. In previous studies, it was reported that a microtubule interaction is essential for Survivin's anti-apoptotic function in mitosis [10]. Our observations render such models less likely.

In summary, we have reported that vertebrate Survivin is a chromosomal passenger protein that can interact directly with Aurora-B and INCENP and whose targeting to centromeres and the central spindle depends on INCENP. Furthermore, as it was previously shown that INCENP exists in a complex with Aurora-B [7, 9], all combinations of interaction are possible within this triad of proteins. The prevailing cytokinesis defect that is manifest when the function of any one of these proteins is impaired may reflect a disruption of a chromosomal passenger complex.

Supplementary material

Supplementary material including a full description of the materials and methods is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

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References

1. Eckley DM, Ainsztein AM, Mackay AM, Goldberg IG, Earnshaw WC: **Chromosomal proteins and cytokinesis: patterns of cleavage furrow formation and inner centromere protein positioning in mitotic heterokaryons and mid-anaphase cells.** *J Cell Biol* 1997, **136**:1169-1183.
2. Mackay AM, Ainsztein AM, Eckley DM, Earnshaw WC: **A dominant mutant of inner centromere protein (INCENP), a chromosomal protein, disrupts prometaphase congression and cytokinesis.** *J Cell Biol* 1998, **140**:991-1002.
3. Terada Y, Tatsuka M, Suzuki F, Yasuda Y, Fujita S, Otsu M: **AIM-1: a mammalian midbody-associated protein required for cytokinesis.** *EMBO J* 1998, **17**:667-676.
4. Fraser AG, James C, Evan GI, Hengartner MO: ***Caenorhabditis elegans* inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis.** *Curr Biol* 1999, **9**:292-301.
5. Speliotes EK, Uren A, Vaux D, Horvitz HR: **The Survivin-like *C. elegans* BIR-1 protein acts with the Aurora-like kinase AIR-2 to affect chromosomes and the spindle midzone.** *Mol Cell* 2000, **6**:211-223.
6. Severson AF, Hamill DR, Carter JC, Schumacher J, Bowerman B: **The Aurora-related kinase AIR-2 recruits ZEN-4/CeMKLP1 to the mitotic spindle at metaphase and is required for cytokinesis.** *Curr Biol* 2000, **10**:1162-1171.
7. Kaitna S, Mendoza M, Jantsch-Plunger V, Glotzer M: **Incenp and an Aurora-like kinase form a complex essential for chromosome segregation and efficient completion of cytokinesis.** *Curr Biol* 2000, **10**:1172-1181.
8. Adams RR, Carmena M, Earnshaw WC: **Chromosomal passengers and the (Aurora) ABCs of mitosis.** *Trends Cell Biol* 2001, **11**:49-54.
9. Adams RR, Wheatley SP, Gouldsworthy AM, Kandels-Lewis SE, Carmena M, Smythe C, *et al.*: **INCENP binds the Aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavage furrow.** *Curr Biol* 2000, **10**:1075-1078.
10. Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, *et al.*: **Control of apoptosis and mitotic spindle checkpoint by Survivin.** *Nature* 1998, **396**:580-584.
11. Li F, Ackermann EJ, Bennett CF, Rothermel AL, Plescia J, Tognin S, *et al.*: **Pleiotropic cell-division defects and apoptosis induced by interference with Survivin function.** *Nat Cell Biol* 1999, **1**:461-466.
12. Earnshaw WC, Bernat RL: **Chromosomal passengers: towards an integrated view of mitosis.** *Chromosoma* 1990, **100**:139-146.
13. Uren AG, Wong L, Pakusch M, Fowler KJ, Burrows FJ, Vaux DL, *et al.*: **Survivin and the inner centromere protein INCENP show similar cell-cycle localization and gene knockout phenotype.** *Curr Biol* 2000, **10**:1319-1328.
14. Skoufias DA, Mollinari C, Lacroix FB, Margolis RL: **Human Survivin is a kinetochore-associated passenger protein.** *J Cell Biol* 2000, **151**:1575-1582.
15. Cooke CA, Heck MMS, Earnshaw WC: **The INCENP antigens: movement from the inner centromere to the midbody during mitosis.** *J Cell Biol* 1987, **105**:2053-2067.
16. Andreassen PR, Palmer DK, Wener MH, Margolis RL: **Telophase disk: a new mammalian mitotic organelle that bisects telophase cells with a possible function in cytokinesis.** *J Cell Sci* 1991, **99**:523-534.
17. Schumacher JM, Golden A, Donovan PJ: **AIR-2: an Aurora/Ipl1-related protein kinase associated with chromosomes and midbody microtubules is required for polar body extrusion and cytokinesis in *Caenorhabditis elegans* embryos.** *J Cell Biol* 1998, **143**:1635-1646.
18. Ainsztein AM, Kandels-Lewis SE, Mackay AM, Earnshaw WC: **INCENP centromere and spindle targeting: identification of essential conserved motifs and involvement of heterochromatin protein HP1.** *J Cell Biol* 1998, **143**:1763-1774.
19. Adams RR, Maiato H, Earnshaw WC, Carmena M: **Essential roles of *Drosophila* INCENP and aurora-B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction and chromosome segregation.** *J Cell Biol*, in press.
20. O'Connor DS, Grossman D, Plescia J, Li F, Zhang H, Villa A, *et al.*: **Regulation of apoptosis at cell division by p34cdc2 phosphorylation of Survivin.** *Proc Natl Acad Sci USA* 2000, **97**:13103-13107.
21. Wheatley SP, Kandels-Lewis SE, Adams RR, Ainsztein AM, Earnshaw WC: **INCENP binds directly to tubulin and requires dynamic microtubules to target to the cleavage furrow.** *Exp Cell Res* 2001, **262**:122-127.